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14-3-3 Antagonizes Ras-Mediated Raf-1 Recruitment to the Plasma Membrane To Maintain Signaling Fidelity

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We have investigated the role that S259 phosphorylation, S621 phosphorylation, and 14-3-3 binding play in regulating Raf-1 activity. We show that 14-3-3 binding, rather than Raf-1 phosphorylation, is required for the correct regulation of kinase activity. Phosphorylation of S621 is not required for activity, but 14-3-3 binding is essential. When 14-3-3 binding to conserved region 2 (CR2) was disrupted, Raf-1 basal kinase activity was elevated and it could be further activated by ^{V12,G37}Ras, ^{V23}TC21, and ^{V38}R-Ras. Disruption of 14-3-3 binding at CR2 did not recover binding of Raf-1 to ^{V12,G37}Ras but allowed more efficient recruitment of Raf-1 to the plasma membrane and stimulated its phosphorylation on S338. Finally, ^{V12}Ras, but not ^{V12,G37}Ras, displaced 14-3-3 from full-length Raf-1 and the Raf-1 bound to Ras. GTP was still phosphorylated on S259. Our data suggest that stable association of Raf-1 with the plasma membrane requires Ras-mediated displacement of 14-3-3 from CR2. Small G proteins that cannot displace 14-3-3 fail to recruit Raf-1 to the membrane efficiently and so fail to stimulate kinase activity.

The Ras/Raf/MEK/ERK signaling pathway is a conserved signaling module that regulates complex cellular functions such as proliferation, differentiation, cell death, and T-cell activation (for reviews, see references 37 and 49). This pathway consists of a kinase cascade that is activated in a Ras-dependent manner. The first kinases in the cascade are the Raf serine/threonine-specific protein kinases. Active Raf proteins phosphorylate and activate the dual-specificity MEK protein kinases, which in turn phosphorylate and activate the serine/threonine-specific extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases. ERKs phosphorylate and regulate the activity of proteins in the cytosol and the nucleus. The Raf proteins therefore couple Ras signaling to ERK activation. In mammals there are three Raf genes (Raf-1, A-Raf, and B-Raf), and comparison of their protein sequences reveals three conserved regions, conserved region 1 (CR1), CR2, and CR3 (22, 35). CR1 and CR2, found within the N-terminal half of the proteins, appear to be regulatory, while the kinase domain, encapsulated within CR3, is in the C-terminal half of the proteins.

Raf regulation has been the subject of intense scrutiny (for reviews, see references 3, 27, and 43). Raf proteins bind to activated Ras (Ras-GTP) with high affinity through a region called the Ras binding domain (RBD) which is within CR1. Ras is anchored to the inner surface of the plasma membrane and in resting cells, Raf proteins are cytosolic. However, in the presence of Ras-GTP, the Raf proteins are recruited to the plasma membrane, where activation occurs through a mechanism involving phosphorylation, dimerization, binding to other proteins and lipid interactions. Two amino acids whose phosphorylation at the plasma membrane is critical for Raf-1 activity are serine 338 (S338) and tyrosine 341 (Y341), which are in a region that we call the N region (for negative-charge regulatory region) (15, 17, 38).

One family of proteins that interact with Raf-1 are the 14-3-3 adaptor-scaffold proteins. These are highly conserved acidic proteins with molecular masses of ~30 kDa that bind to a large number and variety of client proteins (see references 2 and 46). The binding of 14-3-3 to client proteins occurs through short peptide motifs. For some peptides, binding occurs only if a specific serine within the motif is phosphorylated, but binding to other motifs is phosphorylation independent (45, 47, 61). An emerging concept in 14-3-3 biology is that they bind to and sequester client proteins into inappropriate subcellular compartments, thereby suppressing client protein activity (see reference 46). For example, phosphorylation dependent binding of 14-3-3 to the transcription factor forkhead blocks its ability to repress transcription by removing it from the nucleus (7, 48). Similarly, phosphorylation-dependent binding of 14-3-3 to the proapoptotic protein BAD blocks apoptosis by displacing BAD from mitochondria (11).

The role that 14-3-3 binding plays in regulating Raf-1 is controversial. Many studies suggest that 14-3-3 binding is essential for kinase activity (19, 20, 23, 31, 39, 52, 56, 62), while others suggest that it is not (21, 40, 55). In part, the confusion stems from the fact that there are two 14-3-3 binding sites on Raf-1 that appear to play opposing roles. Both sites conform to the consensus sequence RSXpSXP (single amino acid code [24]: pS, phosphorylated serine; X, any amino acid) (Fig. 1) with binding being dependent on phosphorylation of the central serine (45, 61). One motif is in CR2 and requires phosphorylation of serine 259 (S259) (Fig. 1). The other, in CR3, is at the C-terminal end of the kinase domain and requires phosphorylation of serine 621 (S621) (Fig. 1). Binding of 14-3-3 to CR2 appears to suppress Raf-1 activity, whereas binding to CR3 appears to be essential. Thus, since 14-3-3 proteins are dimeric and can simultaneously bind to two peptides (47, 61), one model suggests that one 14-3-3 dimer binds to both CR2

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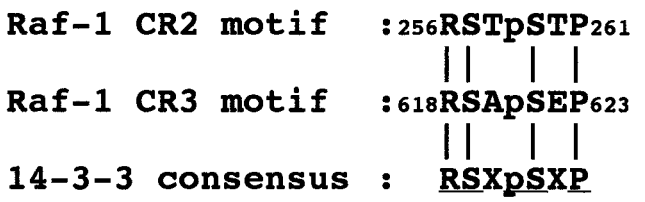


FIG. 1. 14-3-3 binding motifs in Raf-1. The amino acid sequences surrounding the 14-3-3 binding motif in CR2 and CR3 of Raf-1 are shown, together with the consensus motif (45). The single amino acid code is used (X, any amino acid; pS, phosphorylated serine), and amino acids whose positions cannot be altered are underlined in the consensus sequence. Vertical lines indicate conserved residues, and the numbers 256, 261, 618, and 623 refer to amino acid positions in Raf-1.

and CR3 to keep Raf-1 in a closed, inactive conformation (51, 57). Activation requires release of CR2, and 14-3-3 can then bind to a third, unidentified site to maintain the active conformation (see reference 3). Recent studies have shown that protein kinase B can suppress Raf-1 activity by directly phosphorylating S259 (50, 63), and it has also been suggested that protein phosphatase 1 (PP1) and 2A (PP2A) mediate S259 dephosphorylation as a prerequisite for Raf-1 activation in growth factor-stimulated cells (1, 26). It has also been suggested that 14-3-3 dimers bridge Raf-1 to substrates or to other signaling molecules (6, 10).

There also appears to be competition between Ras and 14-3-3 for binding to Raf-1. Both Ras and 14-3-3 have secondary binding sites within the cysteine-rich domain (CRD), which is also in CR1 and C terminal to the RBD (9, 40, 60). In vitro, Ras-GTP displaces 14-3-3 from the isolated N terminus of Raf-1 (51), possibly due to competition for this second site (12). Genetic evidence from yeast two-hybrid analysis using *Saccharomyces cerevisiae* also suggests competitive binding. Raf-1 binds to oncogenic Ras (^{V12}Ras) in yeast cells, but binding is disrupted when glutamate 37 (E37) in Ras is replaced with glycine (^{V12,G37}Ras) (59). Intriguingly, mutations to arginine 256 (R256) or serine 257 (S257) of Raf-1, both of which are within the RSXpSXP motif at CR2, restored Raf-1 binding to ^{V12,G37}Ras in yeast (59) but not in solution (25). These mutations would be expected to disrupt 14-3-3 binding to CR2, but this has not been tested. The ability of ^{V12,G37}Ras to activate Raf-1 in which S257 was replaced with leucine has not been tested directly, but when coexpressed, these proteins activate ERK in cells and stimulate reporter gene expression, something that they cannot do when expressed alone (25, 59).

Finally, it is difficult to analyze the role that 14-3-3 binding to CR3 plays, because S621 mutations are inactivating (4, 44). In one study, it was suggested that Raf-1 activation requires 14-3-3 displacement from CR3 and S621 dephosphorylation (42). It was suggested that S621 mutations are inactivating because the dephosphorylated serine performs a specific function(s) that is required for activity. This model also suggests that 14-3-3 is completely displaced from active Raf-1, and in some experimental conditions, this appears to be the case (52). However, a number of other studies argue that binding of 14-3-3 to CR3 is essential for activity, with the strongest evidence coming from peptide displacement studies. Active Raf-1 can be inactivated by phosphopeptides that displace 14-3-3; Raf-1 is reactivated by subsequent addition of recombinant 14-3-3 (39, 56, 57). Importantly, this approach also works with the isolated kinase domain, supporting binding to CR3 as being essential for activity (62). Furthermore, it has been shown that 14-3-3 binding to CR3 protects S621 from dephosphorylation to maintain Raf-1 activity (56) and also that 14-3-3 protects active Raf-1 from PP1- and PP2A-mediated inactivation (13).

In this study, we have further investigated the complex roles played by S259 phosphorylation, S621 phosphorylation, and 14-3-3 binding in regulating Raf-1 activity. We show that the binding of 14-3-3 to Raf-1, rather than S259 or S621 phosphorylation, is the essential event in Raf-1 regulation. We demonstrate that ^{V12,G37}Ras directly activates Raf-1 when 14-3-3 binding to CR2 is disrupted and that this activation requires Ras binding, membrane localization, and S338 phosphorylation. We show that ^{V12}Ras, but not ^{V12,G37}Ras, displaces 14-3-3 from Raf-1 but that the Raf-1 that is bound to Ras-GTP is still phosphorylated on S259. We also demonstrate that TC21 and R-Ras can only activate Raf-1 when 14-3-3 binding to CR2 is disrupted. Our data suggest that 14-3-3 antagonizes Raf-1 recruitment to the plasma membrane to ensure that Raf-1 is not activated in resting cells and cannot be activated by all Ras-related small G proteins.

MATERIALS AND METHODS

Expression vectors. All cloning steps were performed by standard techniques (53). The *myc*-epitope-tagged Raf-1 (mRaf-1) expression vector (pEFm/Raf-1.6) has been described previously (8). It uses the elongation factor 1α (EF1α) promoter for efficient protein expression and fuses a *myc* epitope tag (MEQKLISEEDLGS) onto the Raf-1 N terminus. This tag is recognized by the mouse monoclonal antibody 9E10 (16) and by the rat monoclonal antibody JAC/6 (K. Maycroft, unpublished data). All the point mutations in mRaf-1, as indicated in the present work, were introduced into this vector by using the PCR with sequence verification by automated dideoxy sequencing procedures. The R-18 sequence was also introduced into this vector by PCR. To introduce the R-18 peptide into CR3, codons 621 to 648 of Raf-1 were replaced with codons encoding R-18 (PHCVPRDLSWLDLEANMCLP). To introduce R-18 into CR2, codons 257 to 268 of mRaf-1 were deleted and R-18 codons were fused between codons 256 and 269. The cDNAs for activated TC21 (^{V23}TC21) and activated R-Ras (^{V38}R-Ras) were cloned into pEFplink.6, which also uses the EF1α promoter but does not incorporate a tag onto the N terminus of the protein. The cDNAs for 14-3-3ζ, ^{V12}H-Ras, and ^{V12,G37}H-Ras were cloned into the expression vector pEFHA/Plink.6, which also uses the EF1α promoter but fuses an N-terminal hemagglutinin (HA) epitope tag (MDYPYDVPDYAGS) to the expressed proteins. This tag is recognized by the mouse monoclonal antibody 12CA5.

Cell culture and biochemical techniques. COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed using Lipofectamine (Gibco-BRL Life Technologies). Cell extractions and Raf-1 kinase assays were performed as described previously (33, 34). Western blotting for the *myc* tag and HA tag was performed as described previously using the monoclonal antibodies 9E10 and 12CA5, respectively (33, 34); Western blotting for ERK was performed using polyclonal antibody 122 (29) or monoclonal antibody to phospho-ERK (catalog no. M8159; Sigma). The S338 phospho-specific antibody has been described previously (38), and phospho-Raf (S259) antibody was obtained commercially (catalog no. 9421; New England Biolabs), as was the monoclonal Raf-1 antibody (catalog no. R19120; Transduction Labs). The analysis for complexes between Raf-1 and Ras and between Raf-1 and 14-3-3 was performed essentially as described previously (32) by probing the Western blots for the appropriate proteins as described in the text. Microinjection studies in MDCK cells were performed essentially as described previously (33). Metabolic labeling with [^{32}P]orthophosphate and transfer of radiolabeled mRaf-1 to Immobilon P membranes (Millipore) were performed as described previously (33). The radiolabeled bands were excised and digested with trypsin (catalog no. v5111; Promega), and the peptides were eluted and resolved on crystalline cellulose thin-layer chromatography plates as described previously (36).

RESULTS

14-3-3 binding to CR3, rather than S621 phosphorylation, is required for Raf-1 activity. We first investigated whether S621 phosphorylation or 14-3-3 binding to CR3 was required for Raf-1 activity. For these studies, we transiently expressed mRaf-1 in COS cells together with an HA-epitope-tagged oncogenic version of H-Ras (HA^{V12}Ras) and activated Src (^{F527}Src). Raf-1 kinase activity was measured in an immunoprecipitation-kinase cascade assay using GST-MEK, GST-ERK, and myelin basic proteins as sequential substrates. In agreement with our previously determined data (33, 34), wild-type mRaf-1 had low basal kinase activity and was strongly activated by HA^{V12}Ras and ^{F527}Src (Fig. 2A). However, when S621 was replaced with alanine (m^{A621}Raf-1) to prevent phosphorylation, Raf-1 was inactive (Fig. 2A).

In order to determine whether the activity was lost because S621 phosphorylation was necessary or was lost due to the consequent loss of 14-3-3 binding, we replaced the RSXpSXP 14-3-3 binding motif in CR3 with a phosphorylation-independent binding motif. The peptide R-18, which has been identified in phage display libraries and contains the sequence WLDLE, has been shown to bind to 14-3-3 in a phosphorylation-independent manner (47, 58). We therefore deleted amino acids 621 to 648 of Raf-1 and fused the R-18 peptide to amino acid 620, creating m^{3-18}Raf-1. First, we tested whether the fusion of R-18 to mRaf-1 recovered binding of 14-3-3 to CR3. However, the results of Raf-1–14-3-3 binding studies can be indeterminate because of the two 14-3-3 binding sites in Raf-1. Therefore, these studies were performed in the background of a serine-for-alanine substitution at position 259, which abolishes binding of 14-3-3 to the CR2 motif (45, 61). mRaf-1 constructs were coexpressed in COS cells with HA-epitope-tagged 14-3-3 (HA14-3-3), and the HA14-3-3 was immunoprecipitated with the 12CA5 monoclonal antibody. The immune complexes were examined for bound Raf-1 by Western blotting for the *myc* epitope. As expected, mRaf-1 in which both S259 and S621 were replaced with alanine ($m^{A259,A621}$Raf-1) did not bind to 14-3-3 (Fig. 2B). By contrast, 14-3-3 did bind to $m^{A259,3-18}$Raf-1 (Fig. 2B), indicating that the R-18 peptide did direct 14-3-3 binding to CR3. The R-18 peptide also partially restored (~30%) mRaf-1 kinase activity (Fig. 2A) and also restored Raf-1 activity in vivo, as shown by its effect on endogenous ERK. As described previously, Raf-1 in which S259 is replaced with alanine (m^{A259}Raf-1) has elevated basal kinase activity (1, 63) (also see below). We used the extracts from the 14-3-3 binding experiments to examine the activation of endogenous ERK in COS cells, using an antibody that binds only to the dually phosphorylated, active form of ERK1/2. As shown (Fig. 2C), m^{A259}Raf-1 overexpression led to elevated ERK phosphorylation, a result which was not seen with wild-type mRaf-1. By contrast, $m^{A259,A621}$Raf-1 failed to stimulate ERK phosphorylation under these conditions, whereas $m^{A259,3-18}$Raf-1 did (Fig. 2C). These data demonstrate that Raf-1 can be activated in the absence of S621 phosphorylation, provided that 14-3-3 binds to CR3.

S259 is phosphorylated in ^{L257}Raf-1, but 14-3-3 binding is lost. Using similar approaches, we also examined the role of 14-3-3 binding to CR2. For these studies, we compared wild-type mRaf-1 to two versions of mRaf-1 in which the CR2 motif is mutated: m^{A259}Raf-1 (as described above) and an mRaf-1 with a leucine-for-serine substitution at position 257 (m^{L257}Raf-1). In ^{L257}Raf-1, the first serine in the RSXpSXP motif is mutated, which restores binding of Raf-1 to ^{V12,G37}Ras (59). We first examined how these mutations affected phosphorylation of Raf-1 on S259. COS cells expressing mRaf-1 proteins were metabolically labeled with [^{32}P]orthophosphate, and the radiolabeled mRaf-1 was subjected to two-dimensional tryptic phosphopeptide mapping. Wild-type mRaf-1 produced five major radiolabeled peptides (peptides A to E) (Fig. 3A). In agreement with previously determined data (44), peptide B was absent from maps of m^{A621}Raf-1 and peptide A was absent from maps in which S43 was substituted for alanine (unpublished data); the origin of peptides C and D is unknown. Peptide E was absent from the maps generated from m^{L257}Raf-1 and also from the maps generated from m^{A259}Raf-1 (Fig. 3A). However, the m^{L257}Raf-1 map contained an additional peptide, E', which was absent from all of the other maps (Fig. 3A). E' and E appear to have similar charges, because they migrate the same distance in the electrophoretic dimension, but E' appears to be the more hydrophobic of the two, as it was more mobile in the ascending chromatography (Fig. 3A). These data suggest that E' may be derived from phosphorylation of S259 in m^{L257}Raf-1. To test this directly, we used a phospho-specific antibody that only binds to Raf-1 when S259 is phosphorylated. In Western blot analysis, the pS259 antibody bound to wild-type mRaf-1 and m^{A621}Raf-1 but not to m^{A259}Raf-1 (Fig. 3B). It also bound to m^{L257}Raf-1 (albeit at lower levels) but not to $m^{L257,A259}$Raf-1 (Fig. 3B). These data show that m^{L257}Raf-1 is phosphorylated on S259, although the levels of phosphorylation may be reduced (see Discussion).

We next tested whether these mRaf-1 proteins bound to 14-3-3 in vivo. Once again, in order to simplify the analysis, these studies were performed in the m^{A621}Raf-1 background to prevent confusion arising due to 14-3-3 binding to CR3. M^{A621}Raf-1 efficiently coimmunoprecipitated with HA14-3-3, whereas $m^{L257,A621}$Raf-1 and $m^{A259,A621}$Raf-1 did not (Fig. 3C). Thus, when S259 phosphorylation was prevented as in ^{A259}Raf-1, 14-3-3 binding to CR2 was disrupted. Despite the fact that S259 was phosphorylated in m^{L257}Raf-1, albeit to lower levels, we did not detect any 14-3-3 binding to this mutant (Fig. 3C).

14-3-3 binding to CR2 suppresses the activation of Raf-1 by ^{V12,G37}Ras. We next tested whether these CR2 mutations affected Ras-mediated Raf-1 activation. Since ^{V12}Ras activates Raf-1 more weakly in the absence of ^{F527}Src (33), the protein expression levels and kinase assays were adjusted to allow accurate measurement of lower levels of Raf kinase activity. The basal kinase activity of mRaf-1 was still low in serum-starved cells, but it was strongly activated by HA^{V12}Ras (Fig. 4A, lanes 1 and 2). The level of basal kinase activity of both m^{L257}Raf-1 and m^{A259}Raf-1 was elevated compared to that of wild-type mRaf-1 but was still higher in the presence of HA^{V12}Ras (Fig. 4A, lanes 4, 5, 7, and 8). Next, we tested whether a HA^{V12}Ras in which the glutamate at position 37 was replaced by glycine ($HA^{V12,G37}$Ras) could activate mRaf-1. $HA^{V12,G37}$Ras did not activate wild-type mRaf-1 but activated both m^{L257}Raf-1 and m^{A259}Raf-1, although to lower levels than those achieved by HA^{V12}Ras (Fig. 4A, lanes 3, 6, and 9). The ability of $HA^{V12,G37}$Ras to activate m^{L257}Raf-1 and

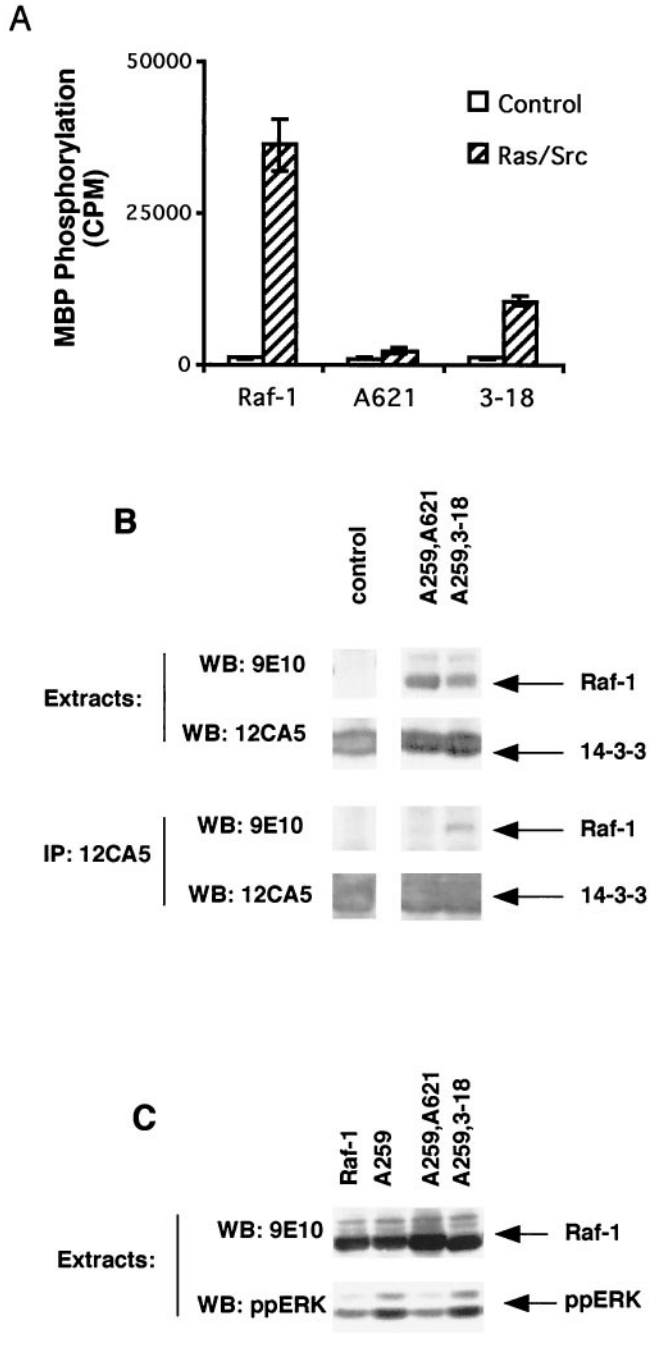


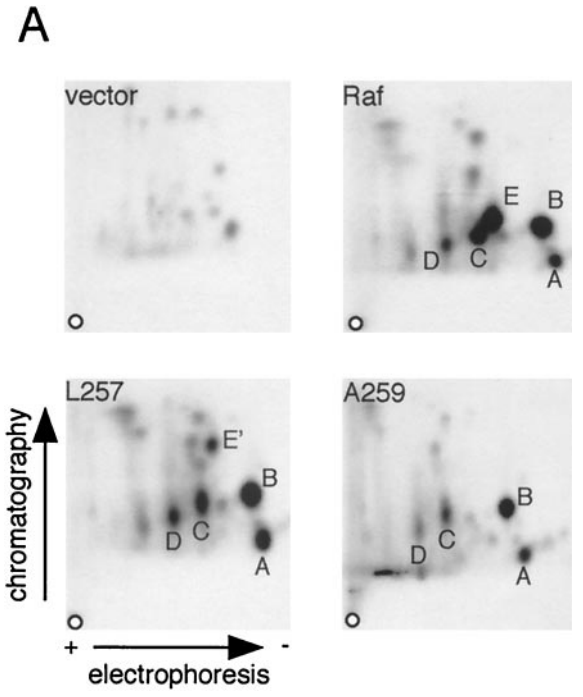
FIG. 2. Binding of 14-3-3 to CR3 of Raf-1 is required for kinase activity. (A) Raf-1 kinase activity. mRaf-1 (Raf), m^{A621}Raf-1 (A621), or m^{3-18}Raf-1 (3-18) was expressed in COS cells together with ^{V12}Ras (Ras) and ^{F527}Src (Src) as indicated, and the cells were serum starved for 24 h prior to extraction. The mRaf-1 proteins were immunoprecipitated for Raf kinase activity determination. The results are from one experiment assayed in triplicate, with error bars representing standard deviations from the means. The background counts (~1,000 cpm) were subtracted, and similar results were obtained in three independent assays. (B) 14-3-3 binding. $m^{A259,A621}$Raf-1 (A259,A621) or $m^{A259,3-18}$Raf-1 (A259,3-18) was expressed in COS cells, together with HA14-3-3. The results for levels of individual protein expression are shown in the upper four panels. HA14-3-3 was immunoprecipitated (IP) using 12CA5, and the samples were resolved on sodium dodecyl sulfate (SDS)–7% polyacrylamide gels and probed by Western blotting (WB) with 9E10 for mRaf-1 and 12CA5 for HA14-3-3 as indicated (lower four panels). Similar results were obtained in three independent assays. (C) Endogenous ERK activation. mRaf-1 (Raf-1), m^{A259}Raf-1 (A259), $m^{A259,A621}$Raf-1 (A259,A621), and $m^{A259,3-18}$Raf-1 (A259,3-18) were expressed in COS cells, together with HA14-3-3. Cell extracts were resolved on SDS–7% polyacrylamide gels and probed by Western blotting (WB) for *myc*-tagged Raf-1 by using 9E10 and for activated endogenous ERK by using a phospho-specific antibody. Similar results were obtained in two independent assays.

m^{A259}Raf-1 in vivo was confirmed by their effects on endogenous ERK. $HA^{V12,G37}$Ras with wild-type mRaf-1 only stimulated very low levels of endogenous ERK phosphorylation, but when $HA^{V12,G37}$Ras was coexpressed with m^{L257}Raf-1 or m^{A259}Raf-1, ERK was strongly phosphorylated (Fig. 4B). (Note that the stimulation of ERK phosphorylation by m^{A259}Raf-1 alone in this experiment appears lower than that seen in Fig. 2C. This is because the sensitivity of the assay was adjusted to allow us to analyze ERK phosphorylation stimulated by ^{V12,G37}Ras in the presence of the Raf-1 mutants.)

These data show that activation of Raf-1 by ^{V12,G37}Ras correlates with loss of 14-3-3 binding rather than loss of S259 phosphorylation. To test this model further, we replaced the RSXpSXP motif in CR2 with the WLDLE motif containing the R-18 peptide (creating m^{2-18}Raf-1) so that 14-3-3 binding to CR2 would be independent of S259 phosphorylation. First, we tested whether 14-3-3 would bind to the CR2 region of m^{2-18}Raf-1, performing these experiments in the context of m^{A621}Raf-1 to simplify the analysis. Whereas $m^{L257,A621}$Raf-1 and $m^{A259,A621}$Raf-1 did not bind to HA14-3-3, $m^{2-18,A621}$Raf-1 bound with similar affinity to that of m^{A621}Raf-1 (Fig. 4C). Thus, the replacement of the RSXpSXP motif in CR2 by the WLDLE motif containing the R-18 peptide recovered 14-3-3 binding to CR2. What is more, the basal kinase activity of m^{2-18}Raf-1 was similar to that of wild-type mRaf-1, and the ability of $HA^{V12,G37}$Ras to activate m^{2-18}Raf-1 was strongly suppressed compared to its ability to activate m^{L257}Raf-1 and m^{A259}Raf-1 (Fig. 4A, lanes 1 to 3 and 10 to 12). Finally, m^{2-18}Raf-1 was less efficient than either m^{L257}Raf-1 or m^{A259}Raf-1 at cooperating with $HA^{V12,G37}$Ras to stimulate activation of endogenous ERK (Fig. 4B).

Activation of ^{L257}Raf-1 requires N-region phosphorylation and membrane recruitment. The above data show that ^{V12,G37}Ras was able to activate Raf-1 when 14-3-3 binding to CR2 was disrupted. We wished to establish how ^{V12,G37}Ras activated these Raf-1 mutants. First, we tested whether S338 phosphorylation was required. $HA^{V12,G37}$Ras did not activate an m^{A259}Raf-1 or m^{L257}Raf-1 in which S338 was replaced by alanine ($m^{A259,A338}$Raf-1 and $m^{L257,A338}$Raf-1, respectively) (Fig. 5A and B). Furthermore, using an S338 phospho-specific antibody (38), we did not detect any S338 phosphorylation on m^{L257}Raf-1 in serum-starved cells, and whereas $HA^{V12,G37}$Ras did not stimulate S338 phosphorylation on wild-type mRaf-1, it did stimulate S338 phosphorylation on m^{L257}Raf-1 (Fig. 5C, lanes 6, 7, and 9).

The above data show that ^{V12,G37}Ras-mediated activation of ^{L257}Raf-1 and ^{A259}Raf-1 requires Ras-mediated recruitment of the Raf-1 proteins to the plasma membrane and S338 phosphorylation, as previously described for wild-type Raf-1 (38). We therefore tested whether ^{L257}Raf-1 and ^{A259}Raf-1 bound



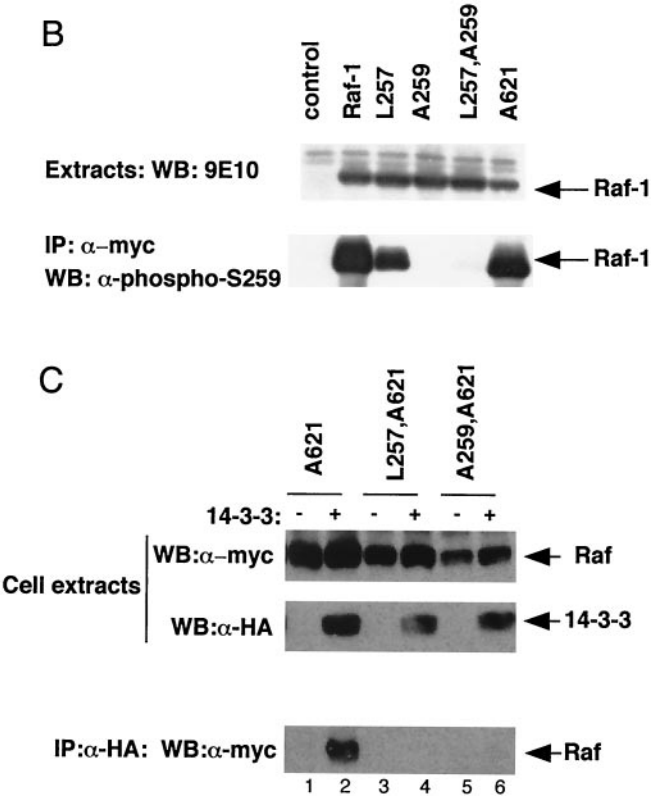


FIG. 3. Binding of 14-3-3 to CR2. (A) Phosphopeptide analysis. COS cells expressing mRaf-1 (Raf), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), or vector control (vector) were metabolically labeled with [^{32}P]orthophosphate, and the radiolabeled mRaf-1 proteins were analyzed by two-dimensional tryptic phosphopeptide mapping. The positions of the five major phosphopeptides are indicated by letters A to E. The directions of the electrophoretic and chromatographic dimensions are indicated. o, origin. (B) Phosphorylation of S259. mRaf-1 (Raf-1), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), $m^{L257,A259}$Raf-1 (L257,A259), or m^{A621}Raf-1 (A621) was expressed in COS cells. The results for levels of individual proteins are shown in the upper panel. mRaf-1 was immunoprecipitated (IP) using 9E10, and the samples were resolved on SDS–7% polyacrylamide gels and probed by Western blotting (WB) for phosphorylation of Raf-1 S259 by using a phospho-specific antibody (lower panel). Similar results were obtained in two independent assays. (C) 14-3-3 binding. Shown are COS cells expressing m^{A621}Raf-1 (A621), $m^{L257,A621}$Raf-1 (L257,A621), or $m^{A259,A621}$Raf-1 (A259,A621) with (+) or without (−) HA14-3-3 (14-3-3) as indicated and processed for 14-3-3 binding as described in the Fig. 2C legend. Results for levels of protein expression are shown in the upper two panels and those for the levels of mRaf-1 associated with HA14-3-3 in the lower panel.

directly to $HA^{V12,G37}$Ras. $HA^{V12,G37}$Ras and mRaf-1 were expressed in COS cells, and the Ras was immunoprecipitated with monoclonal antibody Y13-238. The complexes were examined for coprecipitated mRaf-1 by Western blotting for the *myc* epitope antibody tag. mRaf-1, m^{L257}Raf-1, m^{A259}Raf-1, and m^{2-18}Raf-1 all bound to HA^{V12}Ras strongly (Fig. 6A). However, we did not detect binding of any of these mRaf-1 proteins to $HA^{V12,G37}$Ras (Fig. 6A), suggesting that there was no direct binding to ^{V12,G37}Ras. To test the dependence of direct binding to Ras, we examined the activation of these mutations in a Raf-1 double mutant in which arginine 89 (R89) was also replaced by leucine ($m^{L89,L257}$Raf-1 and $m^{L89,A259}$Raf-1, respectively). R89 is in the Raf-1 RBD, and the replacement of arginine by leucine blocks binding to Ras-GTP, preventing plasma membrane recruitment, S338 phosphorylation, and Raf-1 activation (18, 33, 38). $HA^{V12,G37}$Ras did not activate $m^{L89,A259}$Raf-1 or $m^{L89,L257}$Raf-1 (Fig. 5A and B) and did not stimulate S338 phosphorylation on $m^{L89,L257}$Raf-1 (Fig. 5C, lanes 10 to 12).

Finally, we tested whether $HA^{V12,G37}$Ras could recruit m^{L257}Raf-1 and m^{A259}Raf-1 to the plasma membrane. MDCK cells were microinjected with Ras and Raf-1 expression constructs and the cells were immunostained for Raf-1 with 9E10 or for Ras with Y13-259. In resting cells, both mRaf-1 and m^{A259}Raf-1 were cytosolic, and whereas wild-type mRaf-1 was recruited to the plasma membrane very poorly by $HA^{V12,G37}$Ras, m^{A259}Raf-1 recruitment was very efficient (Fig. 6B). M^{L257}Raf-1 behaved in a manner similar to that of m^{A259}Raf-1; it was cytosolic in resting cells and was efficiently recruited to the plasma membrane by $HA^{V12,G37}$Ras (data not shown), whereas m^{2-18}Raf-1 behaved like wild-type mRaf-1, since its recruitment was very inefficient (Fig. 6B). We did observe that when the cells were incubated for an extended time or the expression levels were high, wild-type Raf-1 was recruited to the plasma membrane by ^{V12,G37}Ras (data not shown), but we cannot confirm that this was due to a direct effect. Taken together, these data show that although disruption of 14-3-3 binding to CR2 does not allow efficient binding of Raf-1 to ^{V12,G37}Ras, ^{V12,G37}Ras was able to recruit Raf-1 to the plasma membrane for S338 phosphorylation and activation when 14-3-3 binding to CR2 was disrupted.

Ras-GTP displaces 14-3-3 from Raf-1, but S259 is still phosphorylated. These data suggest that 14-3-3 binding to CR2 antagonizes Ras-mediated Raf-1 recruitment to the plasma membrane and that wild-type Ras-GTP must therefore displace 14-3-3 from CR2 in order to recruit Raf-1 to the mem-

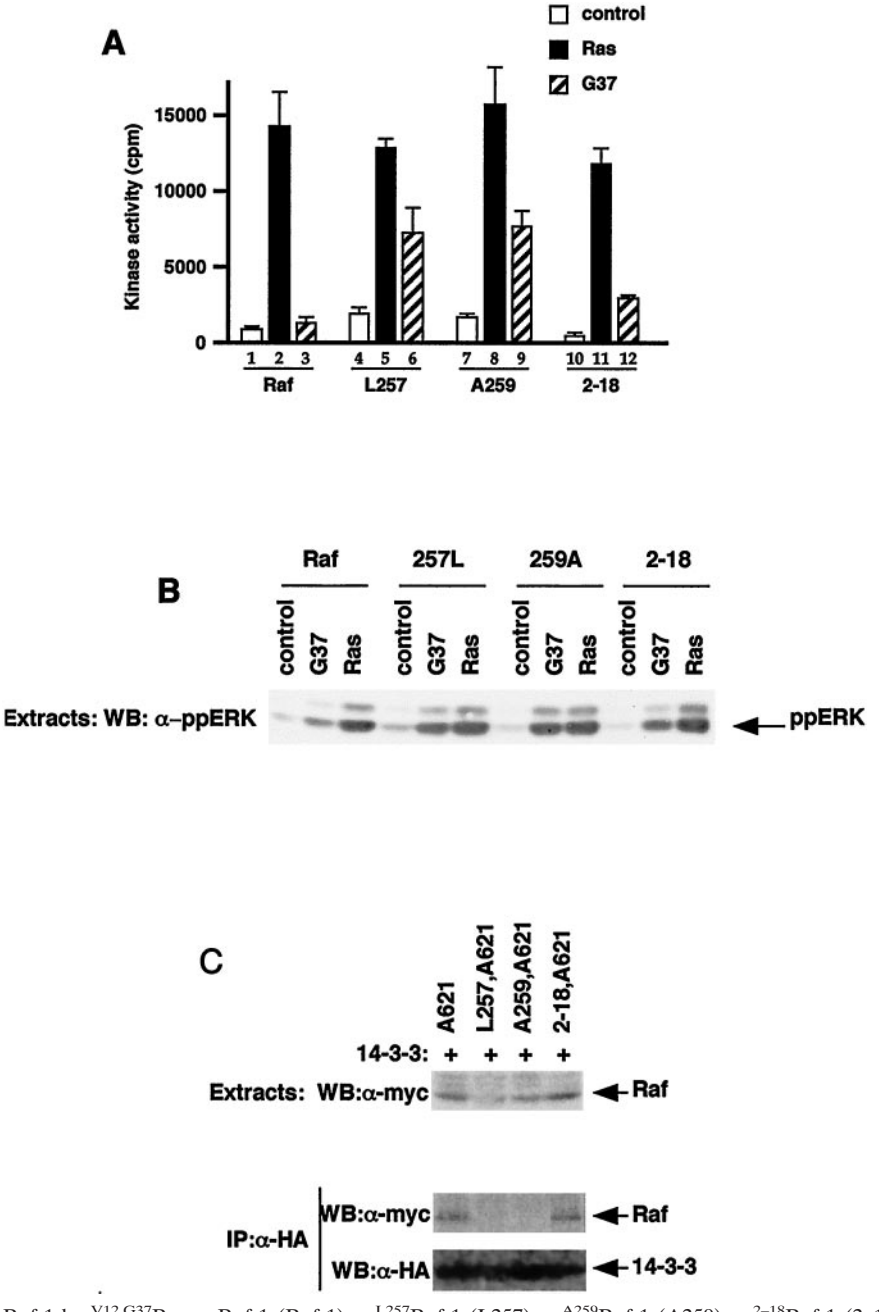
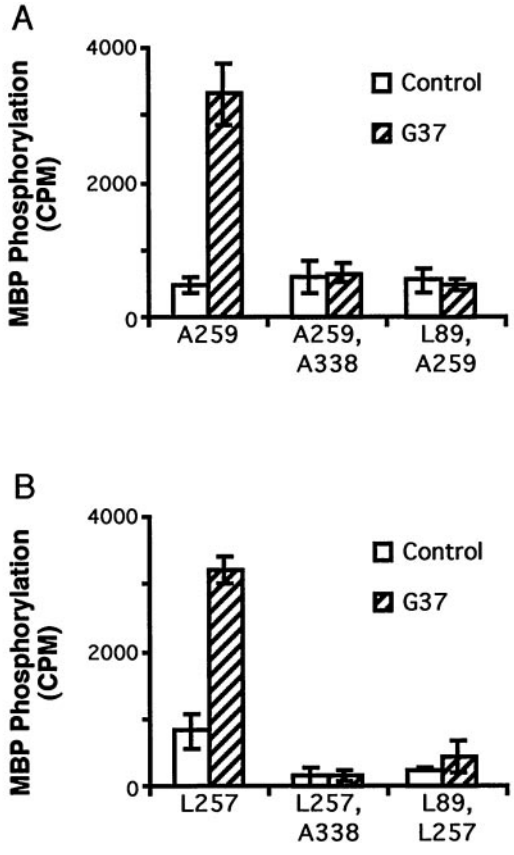


FIG. 4. Activation of Raf-1 by ^{V12,G37}Ras. mRaf-1 (Raf-1), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), m^{2-18}Raf-1 (2–18), m^{A621}Raf-1 (A621), $m^{L257,A621}$Raf-1 (L257,A621), $m^{A259,A621}$Raf-1 (A259,A621), or $m^{2-18,A621}$Raf-1 (2–18,A621) was expressed in COS cells alone (control), with HA^{V12}Ras (Ras), with $HA^{V12,G37}$Ras (G37), or with HA14-3-3 (+) as indicated. (A) Raf-1 kinase activity. Raf-1 kinase assays were performed, and the results are from one experiment assayed in triplicate, with error bars to represent deviations from the means; background counts (~1,445 cpm) were subtracted, and similar results were obtained in three independent experiments. (B) ERK activation. Cell extracts were resolved in SDS–7% polyacrylamide gels and subjected to Western blotting (WB) for activation of endogenous ERKs by using a phospho-specific antibody. Similar results were obtained in three independent assays. (C) 14-3-3 binding. Binding of Raf-1 to HA14-3-3 was determined as described in the Fig. 2C legend. The results for levels of mRaf-1 proteins are shown in the upper panel, and those for levels of the mRaf-1 proteins associated with HA14-3-3 are shown in the middle panel. The results for efficiency of the 12CA5 immunoprecipitate (IP) are shown in the lower panel.

brane. To test this model, mRaf-1 was immunoprecipitated from COS cells by using the rat anti-*myc* antibody JAC/6 and the binding of HA14-3-3 was examined by Western blotting with 12CA5. In resting cells, HA14-3-3 was efficiently immunoprecipitated by JAC/6 in the presence of mRaf-1, indicating strong binding between these proteins (Fig. 7A, lanes 1 and 3). In the presence of HA^{V12}Ras, however, 14-3-3 binding was significantly reduced, whereas in the presence of $HA^{V12,G37}$Ras, it was not (Fig. 7A, lanes 3, 4, and 6). In similar experiments using m^{A259}Raf-1 or m^{L89}Raf-1, no reduction in binding to HA14-3-3 was observed on coexpression of HA^{V12}Ras (data not shown). Thus, as Ras-GTP did displace



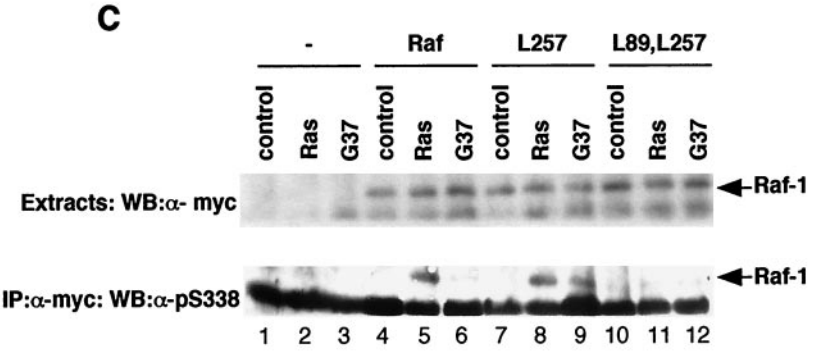
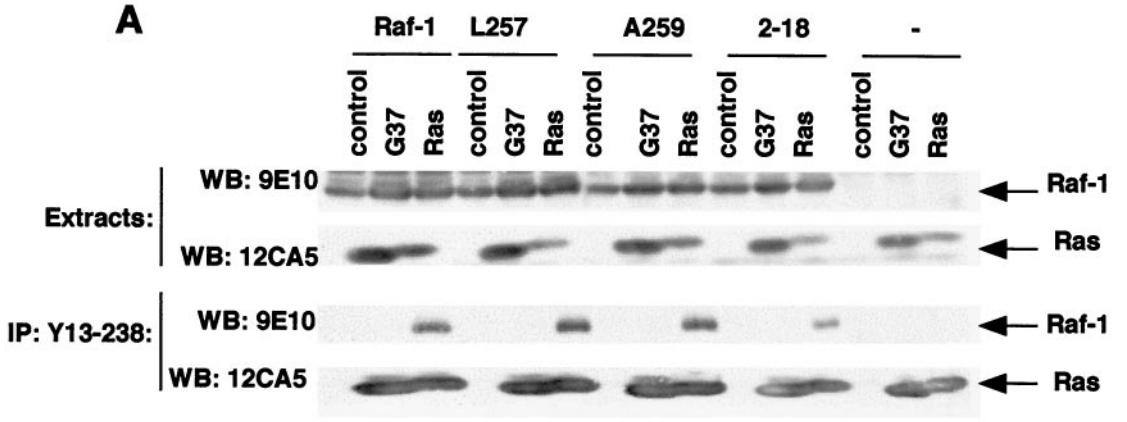


FIG. 5. Raf-1 activity requires S338 phosphorylation. mRaf-1 (Raf), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), $m^{L89,L257}$Raf-1 (L89,L257), $m^{L89,A259}$Raf-1 (L89,A259), $m^{A259,A338}$Raf-1 (A259,A338) or $m^{L257,A338}$Raf-1 (L257,A338) was expressed in COS cells alone (control), with HA^{V12}Ras (Ras), or with $HA^{V12,G37}$Ras (G37) as indicated. (A and B) Raf-1 kinase activity. Raf-1 kinase assays were performed, and the data are shown for one experiment assayed in triplicate with background counts (1,100 cpm) subtracted and error bars to represent standard deviations from the means. Similar results were obtained in three independent assays. (C) S338 phosphorylation. The mRaf-1 proteins were immunoprecipitated (IP), and the levels of S338 phosphorylation were determined using a phospho-specific antibody. The upper panel shows results for the levels of mRaf-1 protein expression, and the lower panel those for the corresponding levels of S338 phosphorylation. WB, Western blotting.

14-3-3 from CR2 in Raf-1, we tested whether the binding of Raf-1 to Ras-GTP was accompanied by S259 dephosphorylation. These experiments were performed on endogenous proteins in COS cells treated with epidermal growth factor (EGF), which activates Ras and stimulates the formation of Ras–Raf-1 complexes (32). These complexes were immunoprecipitated with the Ras monoclonal antibody Y13-238, and the Raf-1 in the complexes was subjected to Western blotting with the



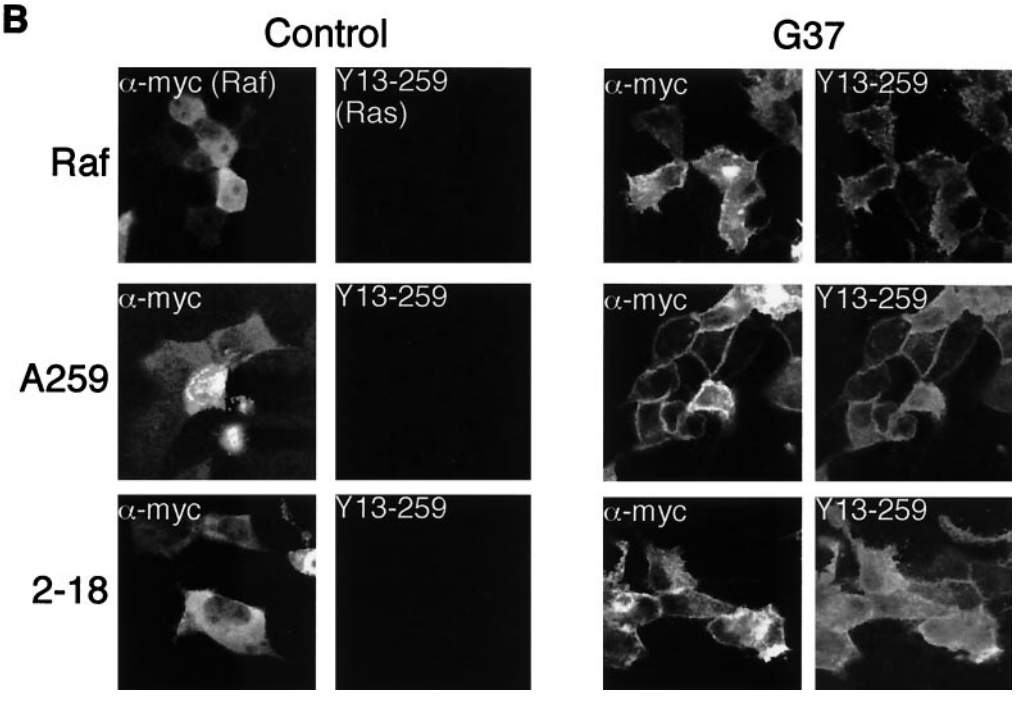


FIG. 6. Membrane recruitment of Raf-1. (A) Ras-Raf binding. COS cells were transfected with expression constructs for mRaf-1 (Raf), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), or m^{2-18}Raf-1 (2–18) alone (control), with HA^{V12}Ras (Ras), or with $HA^{V12,G37}$Ras (G37) as indicated, and the results for levels of protein expression are shown in the upper two panels, as revealed by Western blotting (WB) with 9E10 for mRaf-1 proteins or 12CA5 for Ras. The Ras proteins were immunoprecipitated (IP) with the Y13-238 antibody and the complexes were resolved on SDS–7% polyacrylamide gels, as shown in the lower two panels. Western blotting (WB) with 12CA5 as shown in the bottom panel revealed the efficiency of the Ras precipitation, and the bound mRaf-1 proteins were revealed by Western blotting with 9E10, shown in the lower middle panel. Similar results were obtained in at least three independent assays. (B) Membrane recruitment. MDCK cells were microinjected with expression constructs for mRaf-1 (Raf; top row), m^{A259}Raf-1 (A259; middle row) or m^{2-18}Raf-1 (2–18; bottom row) alone (control; left two columns) or with $HA^{V12,G37}$Ras (G37; right two columns) as indicated. For each sample, the same field of cells, stained for mRaf-1 protein expression (α-*myc*; leftmost and middle right columns) or for Ras expression (Y13-259; rightmost and middle left columns), is shown. Similar results were obtained in three independent assays.

pS259 phospho-specific antibody. S259 was phosphorylated in resting COS cells, and EGF treatment did not significantly alter these levels of phosphorylation (Fig. 7B). Furthermore, the Raf-1 that was bound to Ras was recognized by the S259 phospho-specific antibody, showing that the binding of Raf-1 to Ras-GTP does not require dephosphorylation of S259 (Fig. 7B).

TC21 and R-Ras activate ^{A259}Raf-1 but not wild-type Raf-1.

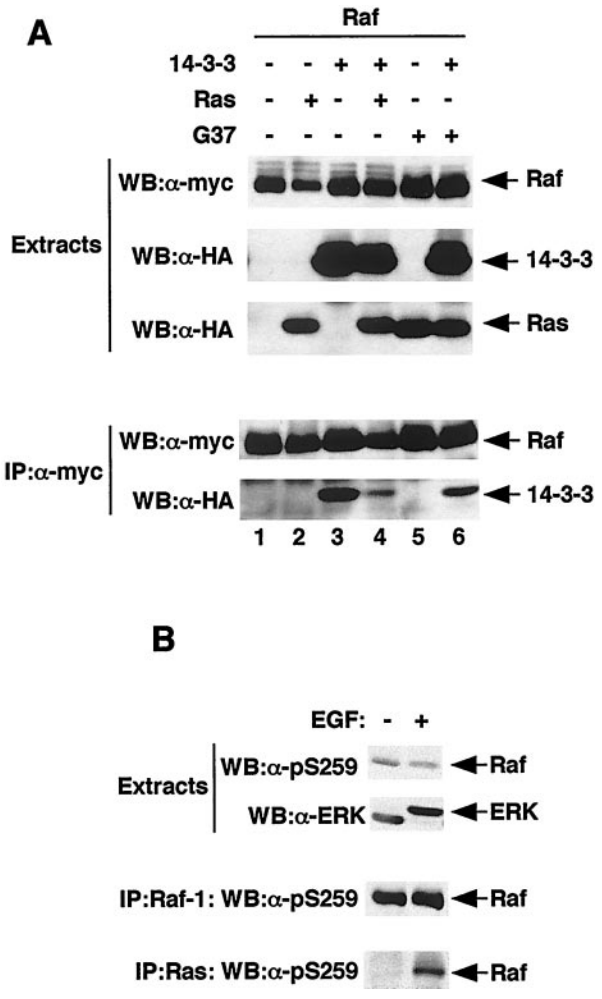


FIG. 7. Displacement of 14-3-3 from Raf-1 by Ras does not require S259 dephosphorylation. (A) 14-3-3 binding. COS cells were transfected with expression constructs for mRaf-1 (Raf), HA14-3-3 (14-3-3), HA^{V12}Ras (Ras), or $HA^{V12,G37}$Ras (G37) as indicated. The levels of protein expression were revealed by Western blotting (WB) for mRaf-1 with 9E10 (top panel of top three) and for HA14-3-3 and HA-tagged Ras protein by Western blotting with 12CA5 (middle and bottom panels of top three, respectively). The mRaf-1 was immunoprecipitated (IP) using the rat monoclonal antibody JAC/6, and the complexes were resolved on SDS–7% polyacrylamide gels and probed for mRaf-1 by using 9E10 (upper panel of bottom two) or for HA14-3-3 by using 12CA5 (lower panel of bottom two). Similar results were obtained in two independent experiments. (B) S259 phosphorylation. COS cells were treated with EGF (10 ng, 20 min), and the levels of pS259 on Raf-1 were examined by Western blotting (WB) with a phospho-specific antibody, either in extracts (upper panel of top two) or following immunoprecipitation (IP) of Raf-1 by using a Raf-1 monoclonal antibody (upper panel of bottom two). As a loading control and to ensure that EGF had activated this pathway, the mobility of endogenous ERK2 in SDS gels was examined using antibody 122 (lower panel of top two). Finally, endogenous Ras was immunoprecipitated, and the levels of S259 phosphorylation on the coprecipitated Raf-1 were determined using the phospho-specific antibody (lower panel of bottom two). Similar results were obtained in three independent experiments.

Finally, we wished to examine the biological consequences of 14-3-3 binding and Raf-1 activation by small G proteins. A number of Ras family members have a very similar effector domain to Ras and bind to Raf-1 in vitro but do not activate Raf-1 (see reference 5). We therefore tested whether these proteins could activate the mutant Raf-1 proteins characterized above. Activated versions of Rap1a, Rap2a, and Rap2b failed to activate any of the mRaf-1 proteins (data not shown). By contrast, whereas activated versions of TC21 (^{V23}TC21) and R-Ras (^{V38}R-Ras) did not activate wild-type mRaf-1, they did activate both m^{L257}Raf-1 and m^{A259}Raf-1, albeit less efficiently than HA^{V12}Ras (Fig. 8A). We also tested the in vivo activity of these proteins by examining their effects on endogenous ERK. When expressed with wild-type mRaf-1, ^{V23}TC21 and ^{V38}R-Ras failed to stimulate endogenous ERK phosphorylation, but they did stimulate ERK phosphorylation when expressed with m^{L257}Raf-1 and m^{A259}Raf-1 (Fig. 8B), indicating that the latter proteins were also activated in vivo. Finally, ^{V23}TC21 and ^{V38}R-Ras failed to activate m^{2-18}Raf-1 (Fig. 8A). Thus, ^{V23}TC21 and ^{V38}R-Ras could activate Raf-1 only when 14-3-3 binding to CR2 was disrupted.

DISCUSSION

Previously it had not been possible to analyze the role played by 14-3-3 binding to CR3 in Raf-1 regulation, because mutations to S621 are inactivating. However, we show that Raf-1 activation can occur in the absence of S621 phosphorylation, provided that 14-3-3 binding to CR3 is maintained. The R-18 peptide provided phosphorylation-independent 14-3-3 binding to CR3 and supported Raf-1 activity in vitro and in vivo, even though S621 was completely absent. We also observed that ^{V12}Ras did not completely displace 14-3-3 from full-length Raf-1 (Fig. 7A), which is a result consistent with the data of others who have suggested that the residual binding is likely to occur at CR3 (28, 39, 51). While our data do not prove that 14-3-3 is bound to CR3 in active Raf-1, they do show that S621 is not absolutely required for activity and so argue that this phosphorylation does not perform an alternative function in the active conformation, as has been suggested (42). Rather, we support the model proposed for peptide displacement and protection from phosphatase-mediated inactivation studies (13, 56, 57, 62), in which 14-3-3 remains bound to CR3 in active Raf-1, where it performs an essential role.

We note that the recovery of activity in $^{3-18}$Raf-1 was only ~30% of that of wild-type Raf-1 (Fig. 2A), suggesting that the R-18 peptide does not replace all functions of the RSXpSXP motif. This may be because 14-3-3 binds to these motif peptides in different ways or because the spacing between 14-3-3 and the kinase domain is slightly compromised in the R-18 fusion. We are presently examining these issues, but nevertheless, our data clearly show that Raf-1 activity can be maintained in the absence of S621 phosphorylation, and this is the first report that demonstrates that it is possible to replace phosphorylation-dependent 14-3-3 binding sites with phosphorylation-independent sites and maintain activity in vitro and in vivo. This may therefore be a useful technique for the study of the role played by 14-3-3 binding in other client proteins, since binding can be made independent of the kinase and phosphatase activity. Studies are ongoing to further examine the role played by 14-3-3 binding to CR3 in Raf kinase activity.

We also examined how 14-3-3 binding to CR2 affects Raf-1 activation mediated by ^{V12,G37}Ras. For those studies, we used two Raf-1 proteins that have mutations in CR2, ^{L257}Raf-1, and

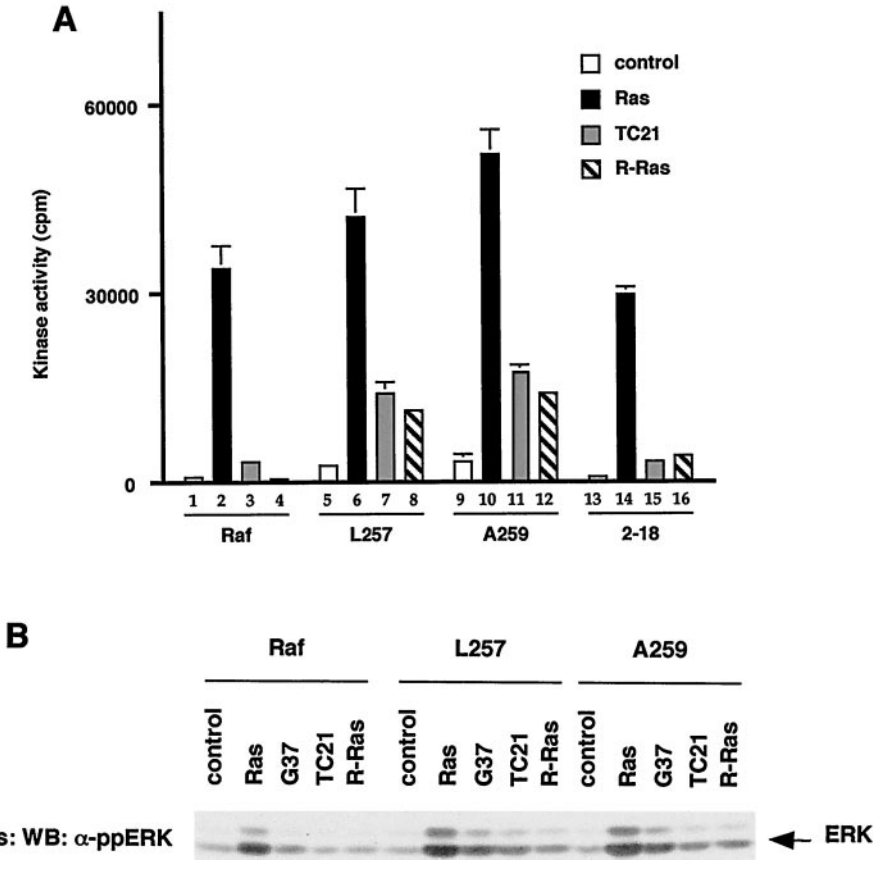


FIG. 8. Activation of Raf-1 by TC21 and R-Ras. mRaf-1 (Raf-1), m^{L257}Raf-1 (L257), m^{A259}Raf-1 (A259), or m^{2-18}Raf-1 (2-18) was expressed in COS cells alone (control) or with HA^{V12}Ras (Ras), $HA^{V12,G37}$Ras (G37), ^{V23}TC21 (TC21), or ^{V38}R-Ras (R-Ras) as indicated. (A) Raf-1 kinase activity. Raf-1 kinase assays were performed, and the results are from one experiment assayed in triplicate, with error bars to represent deviations from the means; background counts (3,164 cpm) were subtracted, and similar results were obtained in two independent experiments. (B) ERK activation. COS cell extracts were resolved on SDS–7% polyacrylamide gels and probed by Western blotting (WB) for activated ERK by using a phospho-specific antibody. Similar results were obtained in two independent experiments.

^{A259}Raf-1. The interaction between 14-3-3 and ^{L257}Raf-1 has not been examined before, although it has been suggested that ^{V12,G37}Ras activates ^{L257}Raf-1 because these proteins interact at the plasma membrane, resulting in a conformation change in the Raf-1 (41). For those studies, however, Raf-1 was anchored to the plasma membrane by fusion of the lipid anchor from Ras (RafCAAX) (30, 54), which leads to constitutive phosphorylation on S338 (an event that occurs at the plasma membrane) (38), so it was not possible to assess the role played by Ras-mediated membrane recruitment or S338 phosphorylation in the activation process. Furthermore, for those studies, reporter gene assays and ERK activation were used as surrogate measures of Raf-1 activity and direct measurements were not made (25, 59).

We showed here that ^{V12,G37}Ras activates ^{L257}Raf-1 and ^{A259}Raf-1 in vitro and in vivo (Fig. 4A) and stimulates S338 phosphorylation on ^{L257}Raf-1. However, ^{V12,G37}Ras did not activate ^{L89,L257}Raf-1 or ^{L89,A259}Raf-1 and did not stimulate S338 phosphorylation on ^{L89,L257}Raf-1 (Fig. 5). Thus, when the RBD of these mutants was disrupted, S338 phosphorylation and activation were blocked, suggesting that they must still interact with ^{V12,G37}Ras and be recruited to the plasma membrane for S338 phosphorylation and activation. Our unpublished data also suggest that ^{L257}Raf-1 and ^{A259}Raf-1 associate with the membrane more readily than wild-type Raf-1. We performed membrane-cytosol fractionation studies and found that even in resting cells, small proportions of ^{L257}Raf-1 and ^{A259}Raf-1 were in the membrane fraction, whereas those of the R89L versions were not (data not shown). Unfortunately, in our hands the results of these experiments were somewhat variable, for reasons that are unclear, but they are consistent with results published while this paper was being reviewed. Dhillon et al. demonstrated that in their hands, a small amount of ^{A259}Raf-1 was in the membrane fraction of resting cells and that ^{V12}Ras recruited ^{A259}Raf-1 to the membrane more efficiently than it recruited wild-type Raf-1 (14). They also demonstrated that ^{L89,A259}Raf-1 is not recruited to the plasma membrane and is not activated.

Together, these studies suggest that ^{L257}Raf-1 and ^{A259}Raf-1 partition to the plasma membrane more readily than wild-type Raf-1 but that this association still requires Ras binding, which is presumably provided by low levels of Ras signaling present in resting cells. Both wild-type Raf-1 and ^{L257}Raf-1 bind to ^{G37}Ras-GTP in vitro with similar but very low affinities (25), whereas in yeast ^{L257}Raf-1 binds to ^{V12,G37}Ras with higher affinity than wild-type Raf-1 (59). We also found a distinction

between the in vivo and in vitro results. Wild-type Raf-1, ^{L257}Raf-1, and ^{A259}Raf-1 failed to bind to ^{V12,G37}Ras in extracts (Fig. 6A), but ^{A259}Raf-1 was still efficiently recruited to the plasma membrane by ^{V12,G37}Ras whereas wild-type Raf-1 was not (Fig. 6B). Thus, the differences in the binding of ^{L257}Raf-1 and wild-type Raf-1 to ^{V12,G37}Ras appear to manifest themselves only in vivo.

We interpret these data with the following model. We propose that Raf-1 recruitment to the plasma membrane requires at least two events. One is the direct binding of Raf-1 to Ras, and the other is neutralization of a negative function mediated by CR2. Wild-type Ras-GTP both binds to Raf-1 and neutralizes the CR2 function. ^{G37}Ras-GTP, by contrast, can bind to Raf-1 but cannot neutralize CR2 and so does not stabilize Raf-1 interaction with the membrane. However, the CR2 function is already neutralized in the ^{L257}Raf-1 and ^{A259}Raf-1 mutants, so that even though the binding to ^{G37}Ras-GTP is weak, a stable association with the membrane can form, allowing S338 phosphorylation and activation. This model predicts that these mutants are very sensitive even to weak Ras signals and so suggests that unlike wild-type Raf-1, the mutants are able to respond to the weak Ras signals in resting cells, which explains why ^{A259}Raf-1 weakly associates with the plasma membrane in these cells (14) and why ^{L257}Raf-1 and ^{A259}Raf-1 have elevated basal activity (Fig. 4). It also explains why the R89L and S338A versions do not have so-called elevated basal activity (Fig. 5A and B), because in our model, the basal activity requires Ras interaction and S338 phosphorylation.

We propose that the function at CR2 that Ras must overcome is 14-3-3 binding, and we propose that Ras-GTP must displace 14-3-3 from CR2 for Raf-1 to form a stable interaction with the plasma membrane, allowing S338 phosphorylation and Raf-1 activation. When the RSXpSXP motif at CR2 was replaced by R-18, the resulting protein was more similar to wild-type Raf-1 than to the CR2 mutants. First, 14-3-3 binding to CR2 was restored (Fig. 4C). Second, the basal activity of Raf-1 was suppressed (Fig. 4A). Third, ^{V12,G37}Ras activation of Raf-1 was strongly suppressed (Fig. 4A). Fourth, the ability of Raf-1 to couple to ^{V12,G37}Ras in vivo and activate endogenous ERK was suppressed (Fig. 4B). Fifth, the ability of ^{V12,G37}Ras to recruit Raf-1 to the plasma membrane was strongly suppressed (Fig. 6B), and sixth, we show that ^{V12,G37}Ras could not displace 14-3-3 from full-length Raf-1, whereas ^{V12}Ras could.

We note that the R-18 peptide replacement was not perfect and that $^{2-18}$Raf-1 had properties that are intermediate between those of wild-type Raf-1 and ^{L257}Raf-1/^{A259}Raf-1; $^{2-18}$Raf-1 was activated by ^{V12,G37}Ras more strongly than wild-type Raf-1, and it coupled to ^{V12,G37}Ras to activate endogenous ERK more strongly than Raf-1. However, the data demonstrate that the binding of 14-3-3 to CR2 suppresses the ability of ^{V12,G37}Ras to be able to achieve efficient recruitment of Raf-1 to the plasma membrane, such that the loss of 14-3-3 binding at this region plays a crucial role in the subsequent activation of Raf-1 and ERK. It has been suggested that Ras and 14-3-3 compete with each other for binding to the CRD of Raf-1, which flanks the RBD and separates it from CR2 (for a review, see reference 3). It is possible that this region affects Ras-mediated 14-3-3 displacement, since both proteins have secondary binding sites in the CRD. A theme growing in importance in 14-3-3 biology is that 14-3-3 proteins regulate the subcellular distribution of client proteins (see reference 46), and our model suggests that 14-3-3 prevents Raf-1 association with the plasma membrane in the presence of weak Ras signals.

Our data also suggest that it is not the phosphorylation of S259 that mediates the negative effect at CR2. We demonstrate that S259 was still phosphorylated in ^{L257}Raf-1, albeit to lower levels (although this may reflect a reduction in antibody binding due to S257 forming part of the antibody epitope), but that the properties of ^{L257}Raf-1 and ^{A259}Raf-1 were indistinguishable. Also, S259 was absent in $^{2-18}$Raf-1, so it could not be responsible for mediating the effects on membrane association and activation. Finally, we show that the Raf-1 bound to Ras-GTP is still phosphorylated on S259. While we cannot determine the stoichiometry of this phosphorylation, it clearly shows that S259 dephosphorylation is not necessary for Raf-1 to form a stable interaction with Ras. It has recently been suggested that S259 dephosphorylation is necessary to allow Raf-1 to bind to Ras-GTP (1, 26). However, this suggestion is counterintuitive, because the phosphate on the serine of peptides bound to 14-3-3 is deeply buried and would be inaccessible to phosphatases (61). Therefore, 14-3-3 displacement must precede dephosphorylation, and we propose that Ras-GTP performs this displacement, a model which is in agreement with the demonstration that Ras-GTP can displace 14-3-3 from the isolated N terminus of Raf-1 (51). Subsequent dephosphorylation of S259 may play a role in sustaining Raf-1 activity under some circumstances.

Finally, our model proposes that one of the important biological consequences of 14-3-3 binding to CR2 is that it prevents Raf-1 from being activated by all Ras family members. We show that activated versions of TC21 and R-Ras, despite having the same effector binding domain as Ras, do not activate wild-type Raf-1, but do activate ^{L257}Raf-1 and ^{A259}Raf-1 (Fig. 8). Again, we attribute this difference in activation levels to 14-3-3 binding, since these proteins were largely unable to activate $^{2-18}$Raf-1. It will be interesting to determine which aspects of TC21 and R-Ras prevent them from being able to activate wild-type Raf-1.

In conclusion, we propose that 14-3-3 binding to CR2 of Raf-1 prevents it from being activated by weak Ras signals in resting cells and from being inappropriately activated by all members of the Ras superfamily, despite the fact that these proteins share very similar effector binding domains. We propose that Ras must displace 14-3-3 from CR2 in order to be able to stabilize Raf-1 association with the plasma membrane and allow phosphorylation and activation. Finally, we suggest that S259 dephosphorylation is not required for the displacement of 14-3-3, although it may have a role in regulating activation under other circumstances.

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